Int. J. Dev. Biol. 45: 39-50 (2001)

Developmental Biology of amphibians after Hans Spemann in Germany

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Introduction

Several scientists in Germany during the fifties till the eighties considered the time after Hans Spemann as a period without much success to solve the questions, which were formulated by the famous organizer experiment of Hans Spemann and Hilde Mangold (Spemann and Mangold, 1924). They spoke of a so-called decline of the Freiburg school, which they considered to be correlated with the intrinsic limitation of the techniques, which were essentially extirpation, transplantation and explantation. Also Viktor Hamburger at the first glimpse seems to have held this view in his book "The heritage of Experimental Embryology" (Hamburger, 1988). As a PhD. candidate and later instructor and untenured faculty member (Privatdozent) in Spemann's Zoological Institute at the University of Freiburg, his estimations of Spemann's views must be seriously taken into account. He stated that "the ultimate cause of its (experimental embryology) decline was rooted deeply in its own axiomatic beliefs and its basic frame of reference". It was built on an organismic, holistic view of embryos and their development. "It was inevitable that a forceful assertation of reductionist trends would shake its foundations. Indeed the radical shift of emphasis to the cellular and subcellular levels and, from the 1950's on, to the molecular level, transformed experimental embryology to developmental biology". The term "decline" of experimental embryology was falsely interpreted by some authors as a decline of the Freiburg school. That this opinion is wrong gets clear if we have a look at many recent publications, which used the Einsteck-experiment not only as an important tool but also as the key experiment for the study of molecular mechanisms including gene activation, gene regulation and embryonic axis (pattern) formation . It is true that the role of genes during development was not a central topic in the thoughts of Spemann, although his xenoplastic transplantations between salamander and frog indicated the importance of genes in the reacting tissue (Spemann and Schotte, 1932; Spemann, 1936). With this opinion he was not alone. Other outstanding embryologists including M. Child, Ross Harrison and Albert Dalq also held the belief that the two disciplines, experimental embryology and genetics, had little in common.

On the other hand the organismic holistic view of Spemann later replaced by reductionist trends in cell biology and molecular biology cannot be considered as a dead-end thinking of Spemann. In fact alternative methods were not available at that time. It must be pointed out that the extreme form of reductionist trends, i.e. extensive studies of the DNA and the single cells only in the fifties was supplemented (not replaced) by more holistic views of the whole embryo and organism, including cell-to-cell interactions and pattern formation in the following decades and molecular comparative studies of different species of all 7 animal phyla.

Furthermore Spemann himself initiated the first steps, which turned from the so-called experimental embryology to the direction of the (bio)chemical analysis of early embryonic development, today described as developmental and molecular biology.

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0214-6282/2001/$25.00

Printed in Spain
www.ijdb.ehu.es

Prof. Hans Engländer (left) on his 80th birthday in 1995. *He was my thesis advisor at the Zoological Institute of the University of Cologne prior to my postdoc time in Prof. Tiedemann's lab in Berlin. He published several interesting papers about heterogeneous inducers.*

Spemann's experiment with minced organizer tissue, which showed weak neuralizing activity was the first indication that chemical factors are responsible for the inductive processes. Together with Bautzmann, Holtfreter and Mangold, he reported the inducing activity of the organizer by killing the organizer by crushing, freezing, drying and treatment with ethanol (Bautzmann *et al.*, 1932). Holtfreter and others continued this work by testing also other tissues from different sources (liver, kidney of adult mice etc.). Holtfreter could show that coagulated chick embryo extract was an effective mesoderm inducer (Holtfreter, 1933a, 1933b). These reports were the basis for isolation of inducing factors by the groups of Yamada, Toivonen, Saxén and Tiedemann. As will be shown in detail below, only the group of Tiedemann succeeded in isolating the so-called vegetalizing factor (now known as activin) in homogenous form. Later the observations of Holtfreter were extended by Hsiao-hui Chuang, a Ph.D. candidate in Holtfreter's laboratory at the Zoology Department of the University of Munich and after the war, director of the Shanghai Institute of Cell Biology. Simultaneously Toivonen in Helsinki obtained similar results. Chuang's major publications about this topic appeared in 1939 and 1940, and that of Toivonen in 1940 (Toivonen, 1940). Chuang could show that the mesodermalizing activity of adult liver is lost after brief dipping into boiling water (Chuang, 1939, 1940). However the neural inducing activity of the liver was not inhibited by such treatment. Apparently this so-called heterogeneous inducer contains two different activities, a heat-labile mesodermalizing agent and a heat-resistant neuralizing agent. These finding were the basis of the double gradient hypothesis later formulated by Toivonen and Saxén (Toivonen and Saxén, 1955; Saxén and Toivonen, 1962) and supported by the fractionation experiments of the group of Tiedemann (Tiedemann and Tiedemann, 1964).

The search for early embryonic inducing factors

The results of Holtfreter, Chuang and Toivonen indicated that heterogeneous inducers like kidney, liver, and tissue of chicken embryos contained factors with inducing activity. It should be mentioned that adult tissues were chosen, since in contrast to amphibian embryos (at this time Triturus or Rana only) liver, kidney or bone marrow from mice or rat were available in larger quantities.

At this time the chemical nature of inducing factors present in these tissues was absolutely unknown. Earlier works of Brachet suggested that RNA might play a role in neural induction, since ribonuclease treatment abolished the neuralizing activity (Brachet, 1942, 1944). Repeated experiments in 1952 however indicated that the ribonuclease used in the paper of 1942 was contaminated by proteases (Brachet *et al.*, 1952). Treatment of kidney or liver with trypsin or pepsin resulted in an almost complete disappearance of the spinal-caudal activity of kidney (Toivonen and Kusi, 1948). These results suggested that inducing factors may be proteins in nature. Further experiments with ribonuclease treatment of various heterogeneous inducers indicated that the concept of the role of RNA in the induction process was not valid (Engländer, Johnen and Vahs, 1953; Yamada and Takata, 1955). In contrast Niu (Niu 1953, 1955, 1956, 1958) concluded on the basis of experiments with "conditioned medium" that proteins act as a stabilizer, a carrier or an agent to facilitate the entrance of RNA into the cells (for detailed discussion see Saxén and Toivonen, 1962). He cultivated dorsal blastopore lip or posterior parts of the medullary plate in culture medium and placed competent ectoderm into this so called conditioned medium either simultaneously or after 12-16 days of culture period. The ectoderm differentiated into pigment cells, nerve cells and myoblasts. Treatment of the conditioned medium containing the ectoderm with trypsin or ribonuclease caused the loss of inducing activity. Niu (1956) suggested that the prevention of induction by trypsin was caused by a direct effect on the responding ectoderm. The presence of soy bean trypsin inhibitor prevented the loss of the activity. The loss of the inducing activity after ribonuclease treatment of the samples resulting in an inactivation of RNA was the basis for his hypothesis of the importance of RNA in the induction process. This view was not corroborated by other scientists, mainly by Tiedemann (Tiedemann and Tiedemann, 1959). The method of Niu and Twitty (1953) was quite elegant in such that they used dorsal mesoderm as starting material. With our present knowledge the results with the medium "conditioned" by normal inductor suggest that the medium contained a secreted inducing factor but in rather low concentration,

Prof. Heinz Tiedemann (left) and the author during a meeting in Venice in 1970.

which was not optimal for convincing results. More recently De Robertis and co-workers could show that dorsal blastopore lip secretes chordin under *in vitro* conditions (Sasai *et al.*, 1994).

Nearly simultaneously the teams in Nagoya (Yamada and co-workers) and in Heiligenberg (Tiedemann and co-workers) started extensive experiments to isolate inducing factors from liver, kidney tissue and embryonal extract (Yamada and Takata, 1956; Tiedemann and Tiedemann, 1956 a). In different approaches they could show that the inducing factors are protein in nature. Yamada and co-workers isolated nucleoprotein fractions from liver and treated them with ribonuclease, which did not cause the loss of inducing activity. (Yamada, Hayashi and Takata, 1958). In contrast treatment with pepsin abolished the inducing activity (Hayashi, 1958).

Tiedemann's group on the other hand used the phenol extraction method (Tiedemann and Tiedemann, 1956 a). They could show that after fractionation of chick embryonic extract with phenol the inducing factors could be found in the phenol layer (where proteins will be dissolved). Inducing factors as other simple proteins will be not irreversibly denatured by phenol. Nucleic acids and polysaccharides enriched in the aqueous phase did not show any inducing activity (Tiedemann and Tiedemann, 1956 b). These data and later results with trypsin, which inactivates the inducing factors from chicken embryos, clearly showed that embryonic inducing factors are protein in nature (Tiedemann, *et al.*, 1960). At this time this conclusion was indeed of outstanding importance for all later studies. Although today the central role of proteins for cell structure and various regulatory functions is generally accepted, the discussion about the relevance of inducing factors isolated form chicken embryos lasted until the early nineties.

Chicken as a source of inducing factors - an early indication of evolutionary conserved proteins

During many meetings a standard question addressed to Dr. Tiedemann was why he used chicken instead of amphibians as source for inducing factors. However, before the b.c. (before cloning) period, even sophisticated biochemical methods were not suitable to isolate substantial amounts of biologically active proteins from amphibian embryos, which were in the case of Triturus available in limited amounts. The biochemical purification was severely impeded by the contaminating yolk and lipids. For biologist at this time it made no sense to isolate an inducing factor with phenol from chicken embryos, a compound, which was used in the early days to sterilize the floors and instruments in hospitals. They did not believe that a protein after such a treatment remains its biological activity. Another notorious question was why Tiedemann isolated the factors from chicken and used amphibians as test material. The overwhelming majority of scientists could not be convinced that a chicken factor exerts specific inducing activity in amphibian embryos. Today of course it is generally accepted that amphibian totipotent ectoderm either in the Einsteck-experiment or in the animal cap assay is one of the best system to study the function of embryonic (signalling) inducing factors isolated from tissues of all 7 phyla of the animal kingdom. Since blastula and early gastrula ectoderm consist of omnipotent cells, it is comparable to omni- or pluripotent stem cells of the mammalian embryo. Both cell types are now considered as an important source and tool for tissue and organ engineering (Grunz, 1999a; Chan *et al.*, 1999). Therefore amphibian ectoderm also in the future will be a valuable tool to study cell and tissue differentiation comparable to higher vertebrates. With our present knowledge about evolutionary conserved genes and their products we can imagine that Tiedemann's group was quite ahead of the main stream at this time. This view is corroborated by statements of Jonathan Slack in his book "*Egg & Ego*" (Slack, 1999). He raises the question why Tiedemann did not receive the Nobel Prize. One of his arguments describes the opinion of the scientific community at that time quite well: "In an atmosphere in which embryology had fallen out of fashion and the hunt for inducing factors was regarded as hopeless because of non-specificity, attention would only have been paid to a spectacular platform performer."

Prof. Viktor Hamburger (96 years old, right) in his home during the author's visit to St. Louis in 1996.

Isolation of inducing factors in the light of different hypotheses

On the basis of the Spemann-Mangold organizer experiment different groups tried to find factors responsible for the embryonic axis formation. Already by the use of so-called heterogeneous inducers (kidney, bone marrow, liver etc.) could be shown that these tissues induced in competent ectoderm archencephalic, deuterecenphalic or/and spinocaudal tissues. Lehmann in a review acquainted the experimental embryologist with these useful terms, which were already used much earlier by comparative anatomists (Lehmann, 1942). In the light of different experimental approaches two main theories of axis formation were formulated: Nieuwkoop's activation-transformation hypothesis (Nieuwkoop *et al.*, 1952) and the double-gradient hypothesis of Toivonen and Saxén (1955). Nieuwkoop postulated that the presumptive neural plate first is determined to form archencephalic (forebrain) structures and is then converted (transformed) in the posterior part to deuterencephalic (mid- and hindbrain) and spinocaudal (tail area) structures by additional factors. On the basis of recent data Christof Niehrs suggests a modified model (Niehrs, 1999). In the double-gradient theory two inducing factors (neuralizing and mesodermalizing) alone or in different ratios were considered to be

From right to left, Prof. Makoto Asashima, Dr. Sigrun Knöchel, Prof. Walter Knöchel and H.G. *at a meeting in India in 1997 in honor of Prof. Tuneo Yamada, who passed away in 1996. The author stayed as postdoc in Tuneo Yamada's lab at the Oak Ridge National Laboratory, Tennessee in 1971/1972.*

responsible for the anterior-posterior pattern formation (for details see Saxén and Toivonen, 1962). Toivonen and Saxén (1955) could show that guinea – pig liver induced mainly archencephalic (forebrain) tissue, while guinea-pig bone marrow induced preferentially notochord somites and pronephros. Simultaneous action of bone marrow and liver in the implantation test resulted in the formation of deuterencephalic structures in addition to archencephalic and spinocaudal tissues. Heinz Tiedemann and Hildegard Tiedemann at this time started to isolate inducing factors from liver and chicken embryos (Tiedemann and Tiedemann, 1956 a). They could show that deuterencephalic (rhombencephalon and otic vesicles) inducing proteineous factors could be separated by chromatography on DEAE-cellulose into mainly mesodermal and mainly forebrain (with eyes) inducing protein fractions. After recombination of the two factions again deuterencephalic derivatives where induced (Tiedemann and Tiedemann, 1964). These data were in agreement with the double-gradient hypothesis of Saxén and Toivonen.

The purification of the vegetalizing factor - a homologue of activin

Proteins were isolated from the trunks of 11 days old chicken embryos (for details see elsewhere, Tiedemann and Tiedemann, 1957). The vegetalizing factor can be separated from the bulk of proteins by isoelectric focusing (I. P. of the factor in 6 M urea ~ 8,0). The molecular weight of the factor determined by size exclusion chromatography and zone centrifugation was 25-28 kDa. When the factor was dissolved in 50% formic acid, it was completely cleaved into subunits. The molecular weight of the subunit determined by SE (size exclusion) - HPLC in 50% formic acid is 13000 kDa (Geithe *et al.*, 1981; Schwarz *et al.*, 1981).
Vegetalizing factor and probably also neuralizing factor are partially bound to proteoglycans (Tiedemann and Tiedemann, 1993). The activity of these factors is diminished in this low affinity complex. Enzymatic cleavage of the core-proteins of proteoglycans abolishes the inhibition of the inducing activity (Born *et al.,* 1972). The inducing activity will be modulated by the formation of such complexes and could result in an inhibition. On the other hand the inducing activity of the vegetalizing factor for the formation of mesoderm and endoderm could also be enhanced, if the cell surface proteoglycans will bind the vegetalizing factor causing the limitation of the diffusion of the factor from 3 to 2 dimensions. Under these conditions the concentration of the vegetalizing factor could be increased near or close to their high affinity receptors (Richter and Eigen, 1974; Tiedemann *et al.*, 1998). The formation of proteoglycan complexes is not limited to inducing factors. TGFβ's (transforming growth factors β) also form complexes with proteoglycans (Segarini and Seyedin, 1988; Andres *et al.*, 1992; Yamaguchi *et al.*, 1990).

It was shown that the factor could be purified by affinity chromatography on heparin-sepharose (Born *et al.,* 1987). This prompted us to test together with W.L. McKeehan acidic and basic heparin binding growth factors (HBGF-1 and HBGF-2; fibroblast growth factors) for inducing activity (Grunz *et al.*, 1987). Both factors induced mesodermal tissues preferentially of the ventral type. The chemical properties (MW, subunit structure) of the vegetalizing factor are however different from the heparin binding growth factors, but closely related to transforming growth factors - βs (TGFβs). Independently Igor David and co-workers have shown that TGFβ-2 induces muscle (Rosa *et al.*, 1988). We found that TGFβ-1 induces ventral mesodermal tissues including mesothelium, blood cells and endothelium forming capillary - like networks (Knöchel *et al.*, 1987; Born *et al.*, 1987; Grunz *et al.*, 1987). The inducing activity of the TGFβs is however lower than the inducing activity of the vegetalizing factor. The final purification of the vegetalizing factor from chicken embryos was achieved by four consecutive steps of a newly developed RP (reversed phase)-HPLC procedure. The overall purification was about 10^6 times. A factor from calf kidney was isolated by the same procedure. The factor forms a single band in sodium-dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). The molecular mass was estimated to 25-26 kDa. The factor is cleaved into subunits of about 13 kDa after reduction of disulfide bonds with dithiothreitol or mercaptoethanol. The homogeneous factor induces as the crude factor depending on its concentrations (Grunz, 1983) all kinds of mesodermal tissues as well intestine and endoderm – like epithelium. Large complexes of notochord and endodermal tissue are induced at high concentration of the factor (Asashima *et al.*, 1991; Plessow *et al.*, 1990).

In the autumn of 1989 Makato Asashima visited his friends in Berlin and gave a seminar on his so far unpublished experiments concerning the mesoderm inducing activity of activin, originally known as a gonadal protein which stimulates the release of pituitary follicle-stimulating hormone. It is identical with the erythroid differentiation factor. Activin has the same high mesoderm inducing activity as the vegetalizing factor and similar chemical properties. Makoto Asashima and colleagues then found out together with the group in Berlin that the vegetalizing factor has the same erythroid differentiation activity as activin and like activin, is inhibited by follistatin, which has no inducing activity (Asashima *et al.*, 1990; Asashima *et al.*, 1991b). Finally a partial sequence near the C-terminal end of the vegetalizing factor was identified, which is identical with the corresponding activin A sequence, showing that the factor is activin A or an activin A homologue (Tiedemann *et al.*, 1992).

The activins belong to the TGF-β superfamily of proteins. The three-dimensional structure of the TGF-β2 homodimer has been

determined by X-ray diffraction. The structure can serve as a prototype for other members of the TGF-β superfamily including activin β, a homodimer. Eight cysteines in each monomer are clustered in a core region. The dimers are stabilized by an exposed interchain disulfide bond and two identical hydrophobic interfaces (Daopin *et al*., 1992; Schlunegger *et al*., 1992; McDonald *et al*., 1993; Ogawa *et al*., 1992). It is likely for this reason that the biologically active vegetalizing factor (activin β A dimer) can in part be recovered after reduction of the dimer to 13 kDa subunits by an excess of formic acid, when the formic acid is removed

$$HCOOH \rightleftharpoons CO_2 + 2H^+ + 2e^- \quad E'o = -0.42\ V;$$

$$2\ RSH \rightleftharpoons RS - SR + 2H^+ + 2e^- \quad E'o = -0.23\ V.$$

Reduction with mercaptoethanol leads to an irreversible loss of biological activity, probably by randomization of all (interchain and intrachain) disulfide bonds and the formation of mixed disulfide bonds with mercaptoethanol at reoxidation (Tiedemann *et al*., 1995).

Mechanism of action of the vegetalizing factor (activin)

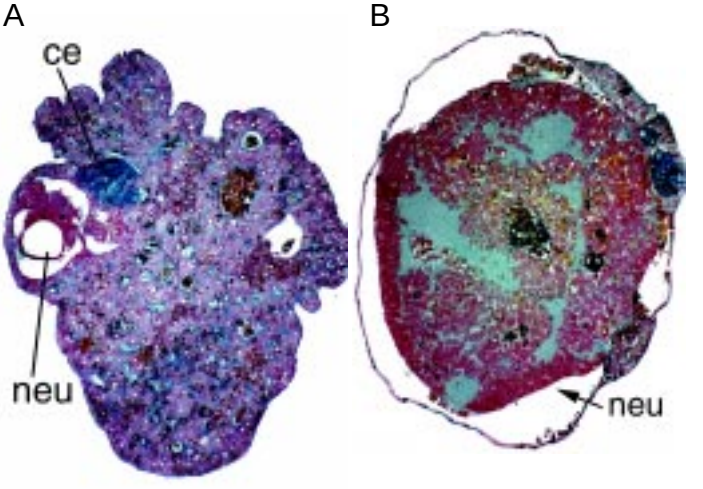
It could be shown by the sandwich-technique (Holtfreter, 1933c) that the mesoderm inducing protein fraction induces also endodermal tissues like gut, liver, pancreas as well as primordial germ cells. It was necessary to culture the induced Triturus alpestris ectoderm up till 6 weeks for a save histological identification. Since all induced tissues are derivatives of the vegetal part of the embryo the factor was called vegetalizing factor (Kocher-Becker and Tiedemann, 1971). When tested at a very high concentration by the Implantation method (Einsteckmethode) the vegetalizing factor causes an exovagination of the gastrula (exovagination should not be confused with exogastrulation). Endoderm, which had invaginated during gastrulation, reappears in the blastopore and spreads over the ectoderm induced to endoderm and mesoderm. The exovagination is caused by a change of cell affinities (Tiedemann *et al*., 1965).

This change of cell affinity after treatment with vegetalizing factor could be confirmed by combination of explants of the vegetal half and mesodermally induced or uniduced animal caps in 1969. Of special interest was the fact that in the control experiments (combination of ectoderm and endoderm) in few cases notochord has differentiated. However, at this time the data were interpreted as a result of a wrong isolation of ectoderm including presumptive notochord. So we omitted these “critical” data and published the paper with considerable delay (Grunz, 1972). Already in 1969 Nieuwkoop published his extensive studies about mesoderm induction by combination of presumptive ectoderm and endoderm, later interpreted as the primary steps for the dorsal mesoderm formation, sometimes called Nieuwkoop center (Nieuwkoop, 1969a, 1969b). It is more likely that in early embryos factors are distributed in a graded distribution (Tiedemann, 1975; Grunz, 1977; Grunz, 1994; Tiedemann *et al*., 1996).

Bone morphogenetic proteins antagonize the organizer’s activity

Inspired by Asashima’s talk in autumn 1989 in Berlin and by the suggestion that activin A or an activin related factor of the TGF-b growth factor family might function as mesodermal inducer, Walter Knöchel started to screen oocyte cDNA libraries using oligonucleotides probes deduced from the activin sequence. This strategy led to the isolation of BMP-2 and BMP-4 (bone morphogenetic protein) cDNAs and the supernatant of BMP-4 transfected COS cells exhibited a weak activity in inducing ventral mesodermal structures in animal cap explants (Köster *et al*., 1991; Plessow *et al*., 1991). This activity became much more obvious, when BMP-4 RNA was injected into the dorsal blastomeres of four-cell stage embryos (Dale *et al*., 1992; Jones *et al*., 1992). Injected embryos were completely ventralized lacking all anterior structures. It was also shown that the ventralizing activity of BMP-4 overrides the dorsalizing activity of activin A. This ventralizing activity corresponds to the spatial zygotic activation of the *BMP-4* gene at late blastula/early gastrula in ventral mesoderm and ectoderm but not within the organizer (Fainsod *et al*., 1994). Moreover, disruption of BMP-signalling by use of a dominant negative truncated BMP type I receptor led to dorsalization and to formation of a secondary axis (Graff *et al*., 1994; Suzuki *et al*., 1994). Thus it became clear that ventralization is a process that is not inherent to the embryo but that must be actively induced by BMP signalling. Due to the fact that BMP-2 and BMP-4 utilize the same receptors, it was subsequently shown that BMP-2 evokes the same ventralizing effects like BMP-4 (Clement *et al*., 1995). However, in contrast to BMP-4, BMP-2 protein is strongly expressed within the mature oocyte. Thus it might be speculated that BMP-2 is a candidate factor involved in primary induction while the function of BMP-4 is mainly required for patterning of the mesoderm. Moreover, BMP-4 signalling is also necessary for the formation of ectoderm (Wilson and Hemmati-Brivanlou, 1995).

A molecular relationship between ventralizing BMP signals and dorsalizing signals secreted by the organizer was discovered by the findings that organizer signals, like chordin or noggin, can bind and inactivate BMP-4 (Piccolo *et al*., 1996, Zimmerman *et al*., 1996). Therefore, it was suggested that one of the primary functions of the organizer is to antagonize the ventral signal and to prevent ventralization (Graff, 1997). Indeed, organizer signals could be shown to be responsible for the establishment of a ventral to dorsal



Results of our dissociation experiments of 1989, which were the basis for the discovery of BMP-4 as a neural inhibitor and antagonist to dorsal factors. (A) *Histological section of ectoderm (40 animal caps) after dissociation into single cells, reaggregated after about 40 minutes and cultured for 5 days. With the exception of a small neural structure (neu), the ectoderm has differentiated into ciliated epidermis, the so-called atypical epidermis (ce, cement gland).* **(B)** *Histological section of ectoderm (40 animal caps) after dissociation, kept as single cells for 4 h prior to reaggregation and cultured for 5 days. The ectoderm has differentiated into large neural structures.*

Volume 300, number 2, 123–126 FEBS 10859 March 1992

The vegetalizing factor

A member of the evolutionarily highly conserved activin family

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Received 9 December 1991; revised version received 11 February 1992

The mesoderm and endoderm inducing vegetalizing factor was partially sequenced after BrCN cleavage. A sequence which is highly conserved in activin A near the C-terminal end was identified. This shows that the factor belongs to the activin family. The activins are not confined to embryos and gonads, but widely distributed in other tissues like calf kidney and calf liver. Functional aspects are discussed.

Embryonic induction; Vegetalizing factor; Activin A; Chicken embryo; Calf kidney; Calf liver

The paper reported the partial sequencing of vegetalizing factor, proving that vegetalizing factor is a member of the activin superprotein family. *After 29 years of tremendous efforts of Prof. Tiedemann and coworkers, it finally could be shown that the vegetalizing factor was the first inducing factor purified to homogeneity. The sequencing was done in the lab of Prof. Lottspeich, the former institute of Nobel Prize winner Prof. Butenandt, in Munich. Therefore all functional studies with this factor ("crude activin") carried out by the author (H.G.) turned out to be as relevant as those using recombinant activin.*

declining BMP-4 protein activity gradient governing dorsoventral gene expression (Dosch *et al.*, 1997). Vice versa, BMP signalling suppresses organizer signals and thus prevents neuralization.

How does BMP-4 signalling suppress dorsal gene activity? This question could be answered by a promoter analysis of the dorsal lip specific early response gene XFD-1´ (Kaufmann *et al.*, 1996). XFD-1´ and its pseudoallele XFD-1 (also known as XFKH1/pintallavis) (Knöchel *et al.*, 1992; Dirksen and Jamrich, 1992; Ruiz i Altaba and Jessell, 1992) are members of the fork head/winged helix multigene family of transcription factors (reviewed in Kaufmann and Knöchel, 1996). XFD-1/1´ gene transcription is directly induced by activin or activin like signals within the organizer. However, this spatial activation is not due to a local activation but to a lack of suppression. Injection of truncated promoter/reporter constructs into ventral blastomeres revealed an inherent capacity of the promoter to be also activated at the ventral side. However, a BMP inhibitory element (BIE) which responds to BMP-2/4 prevents activation of the XFD-1´ gene in the presence of BMP-signalling, thereby allowing its expression only in the organizer. BMP-4 induced inhibition is not direct but mediated by the homeobox factor Xvent-1 (Gawantka *et al.*, 1995) which mimics all the effects of BMP-2/4 on the XFD-1´ promoter and could be shown to bind directly to the previously identified BIE (Friedle *et al.*, 1998). To learn more about the epistatic relationship and regulation of Xvent genes, the genes encoding Xvent-1B and Xvent-2B including their promoters have been analyzed (Rastegar *et al.*, 1999). While Xvent-2B is a direct target of BMP signalling, Xvent-1B is activated by Xvent-2B. Xvent-2 has also been found to activate the BMP-4 gene; thus it is probably involved in the previously reported auto-regulatory loop of BMP-4 expression (Jones *et al.*, 1992). Regulation of the *Xenopus* BMP-4 gene requires factors which bind to the 5´ flanking sequence as well to nucleotide motifs within the second intron (Metz *et al.*, 1998).

The isolation of neuralizing factors

Neuralizing factors remain their inducing activity, when they are covalently bound to bromcyansepharose-beads. The beads are too large for the passage through the plasma membrane (Born *et al.*, 1986). In the intercellular space the factor probably interacts with signal molecules like BMP-4, which inhibit the neuralization of dorsal ectoderm. A low amount of neuralizing factor emanating from the mesoderm could be isolated from the extracellular space between dorsal mesoderm and neural plates of early neurulae (John *et al.*, 1983).

Neural inducing factors could be found in subcellular fractions of *Xenopus* embryos, i.e. ribonucleoprotein-particles (~ 110 A in diameter), which are not identical with subunits of ribosomes and which have a high inducing activity. Furthermore neuralizing factors are found in small vesicles and in the 105 000 x g supernatant. On the other hand plasma membranes contain no neuralizing factor(s) (Bretzel *et al.*, 1986). In the presence of protease inhibitors a neuralizing factor from the supernatant was purified about 1000 fold. Using SDS-polyacrylamide-gel-electrophoresis the main activity could be found in the 13-27 kDa-zone. The neuralizing factor in contrast to the vegetalizing factor will not be inactivated by thioglycolic acid, reducing disulfide-bridges. Treatment with neuraminidase or deglycosylation with trifluoromethansulfonic acid does not irreversibly inactivate the neuralizing factor. This factor is probably a small monomeric protein, part of a larger protein complex, which could be dissociated by SDS (Janeczek *et al.*, 1984; Janeczek *et al.*, 1992; Tiedemann and Tiedemann, 1999). In another approach a partial purified neuralizing factor could be isolated from chicken brain (Mikhailov and Gorgolyuk, 1989; Mikhailov *et al.,* 1995).

The importance of the initial cell mass and secondary cell interactions

In 1983 we could show that different concentrations and incubation times of vegetalizing factor (activin) results in the differentiation of a wide pattern of the treated competent ectoderm (Grunz, 1983). Low concentrations of activin will induce mesenthelium and blood cells, while higher concentrations will cause the differentiation of pronephros notochord and somites. After very high concentrations heart and endodermal structures will be formed. Of great importance for the result is the initial cell mass. If a pellet of vegetalizing factor (comparable to activin bound to sepharose beads, Gurdon *et al.*, 1994) is placed in a single sandwich (2 animal caps), endodermal derivatives will form. A similar pellet placed in a large size sandwich (6-8 animal caps) will cause the differentiation of mesodermal and even neural tissue (Grunz, 1979). These data could show that

different threshold concentrations of the inducing factor triggers the cells in a certain distance to form distinct differentiations. Similar results could be received later using recombinant activin and special genetic markers (Green and Smith, 1990, 1991; Gurdon and Dyson, 1998; Shimizu and Gurdon, 1999). These results were the basis of different hypotheses about the mechanisms of action, the relay mechanism or the long distance diffusion of the inducing factor (Reilly and Melton, 1996; McDowell *et al*., 1997).

Neuralization of disaggregated cells and the neural default hypothesis

In another approach with disaggregated cells we could show that the formation of mesodermal and neural structures is the result of secondary cell interaction. Ectoderm induced by activin (vegetalizing factor) will form mesodermal and neural derivatives. Similar treated ectoderm dissociated into single cells cultured over 20 hours prior to the reaggregation will differentiate into endodermal derivatives and some blood cells. This result showed that mesodermal and neural differentiation are the result of close cell contacts and secondary cell interaction (Minuth and Grunz, 1980). Later we found that untreated *Xenopus* ectoderm, dissociated and kept as single cells for 4 hours prior to the reaggregation, will form neural structures (Grunz and Tacke, 1989). When the extracellular material is added before reaggregation the neural differentiation is prevented. (Grunz and Tacke, 1990). These data later were the basis for the neural default hypothesis of the ectoderm and the identification of BMP-4 as an antagonist to secreted neuralizing factors (Wilson and Hemmati-Brivanlou, 1995; Hemmati-Brivanlou and Melton, 1997). It should be mentioned that first neural specific genes were isolated in the lab of Igor Dawid (Sargent and Dawid, 1983; Richter *et al*., 1988).

New concepts of axis determination

It has been shown that the four animal blastomeres, when isolated from regularly cleaved *Triturus* or *Xenopus* 8-cell stages as a quartet differentiate to mesodermal and neural tissues in over 50 % of the cases (Grunz, 1997, 1994). Other dissection experiments on early cleavage stages came to similar results (Gallagher *et al.,* 1991). This is in accordance with the fate map of *Xenopus* embryos (Nakamura and Kishiyama, 1971). The experiments show that a factor with vegetalizing activity is, after fertilization and cortical rotation prelocalized in the supraequatorial region of the presumptive mesoderm. These factors act by intercellular signalling. Mesoderm is in the embryo obviously not induced in the ectoderm by the presumptive endoderm. There is not an inducing center in the endoderm (Nakamura *et al*., 1970).

Very early during development a shift of vegetal material to the dorsal side takes place by cortical rotation (Gerhart *et al*., 1989). The activation of the Wnt-pathway on the dorsal side will result in the formation of the dorsal mesoderm (Larabell *et al*., 1997). These data indicate that the dorsal vegetal material, sometimes called Nieuwkoop center, can be considered as a precursor area of the Spemann-Mangold organizer. Activin apparently plays an important role for the activation of genes expressed in the dorsal mesoderm. So activin in concert with Wnt, BMP-4 or smad is able to activate siamois, XFKH 1 and mix2 (Harland and Gerhart, 1997; Grunz, 1999b).

Factors in the Spemann-Mangold organizer – inhibitors rather than instructors

It was a big step forward to detect goosecoid (correlated to the *Drosophila* genes gooseberry and bicoid) in the zone of the Spemann-Mangold organizer (Cho *et al*., 1991). This homeobox gene will initiate the expression of Chordin, a secreted protein in the Spemann-Mangold organizer, which will take part in the organization of the central nervous system during gastrulation. A prerequisite to understand the mechanism of action of secreted factors like chordin, noggin, dickkopf and cerberus, which are involved in the formation of the nervous system, was the detection of the central role of BMP-4 expressed on the opposite (ventral) side of the Spemann-Mangold organizer. Genes and their products like *vent-1, vent-2, vox and bmp-4* turned out as anti-organizers. Our main contribution to the new gold rush was our disaggregation experiments of animal caps (Grunz and Tacke, 1989, 1990), which were the basis for the detection of BMP-4 as an anti-neuralizing factor. Disaggregated ectodermal cells will dilute out BMP-4, which results in the formation of brain tissue. Addition of extracellular matrix material (containing BMP-4) or BMP-4 will prevent neuralization and will cause induction of epidermis (Grunz and Tacke, 1990; Wilson and Hemmati-Brivanlou, 1995). On the other hand activin did not shift the determination of ectoderm from neural to epidermis. Even at low concentration of activin dissociated cells differentiated into both neural and mesodermal structures (Grunz, 1996). For detailed discussions of the results the reader is referred to several reviews (Grunz, 1996; Tiedemann *et al*., 1996; Grunz, 1997; Harland and Gerhart, 1997; Tiedemann *et al*., 1998; Grunz, 1999b). On the basis of the results with BMP-4 which prevents neuralization, it has been postulated that the default status of the ectoderm is neural in contrast to the traditional view (Honore and Hemmati-Brivanlou, 1997; Grunz *et al*., 1975; Hemmati-Brivanlou and Melton, 1997).

Cell Differentiation and Development, 28 (1989) 211–218 211
Elsevier Scientific Publishers Ireland, Ltd.

CELDIF 00636

Rapid Communication

Neural differentiation of *Xenopus laevis* ectoderm takes place after disaggregation and delayed reaggregation without inducer

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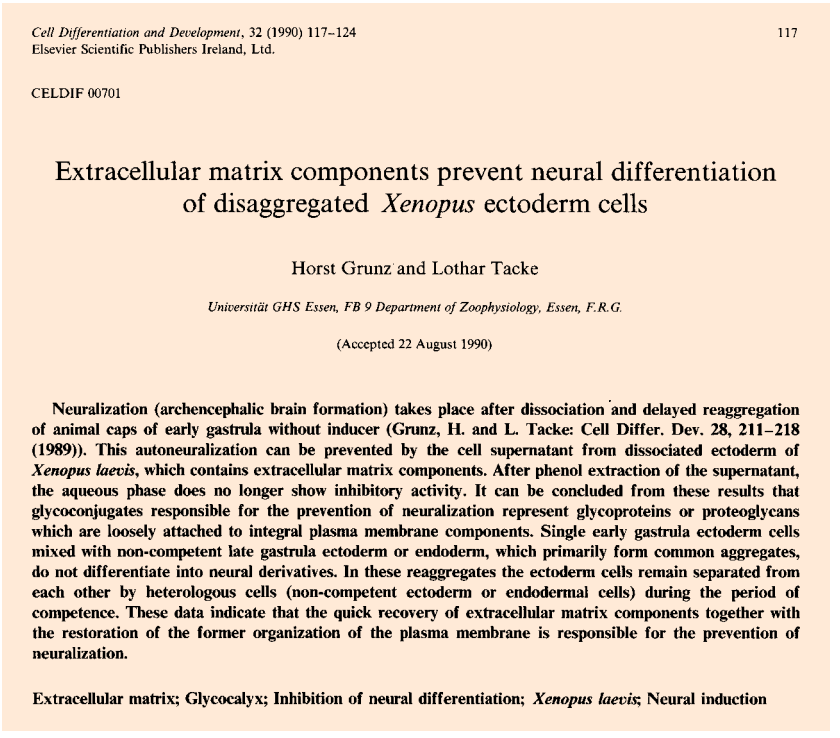
(Accepted 19 October 1989)

When *Xenopus* blastula or early gastrula ectoderm is disaggregated and cells are kept dispersed for up to 5 h prior to reaggregation, the resulting spheres will differentiate into large neural structures. In contrast, dissociated and immediately reaggregated ectoderm will only differentiate into ciliated epidermis (so-called 'atypical epidermis'). Ectoderm treated with mesoderm-inducing XTC-conditioned medium during the period of reaggregation immediately after disaggregation will only form one- or two-cell types (notochord and somites) only. Ectoderm treated with XTC-factor prior to disaggregation will differentiate into a large variety of cell types.

Neural induction; Disaggregation; Induction without external inducer; Mesodermal induction

The paper published in "Cell Differentiation and Development" *(later MOD), which was the basis for the discovery that BMP-4 is responsible for neural inhibition and an antagonist of several neuralizing factors like chordin or cerberus.*



Cell Differentiation and Development, 32 (1990) 117–124
Elsevier Scientific Publishers Ireland, Ltd.

CELDIF 00701

Extracellular matrix components prevent neural differentiation of disaggregated *Xenopus* ectoderm cells

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Neuralization (archencephalic brain formation) takes place after dissociation and delayed reaggregation of animal caps of early gastrula without inducer (Grunz, H. and L. Tacke: Cell Differ. Dev. 28, 211–218 (1989)). This autoneuralization can be prevented by the cell supernatant from dissociated ectoderm of *Xenopus laevis*, which contains extracellular matrix components. After phenol extraction of the supernatant, the aqueous phase does no longer show inhibitory activity. It can be concluded from these results that glycoconjugates responsible for the prevention of neuralization represent glycoproteins or proteoglycans which are loosely attached to integral plasma membrane components. Single early gastrula ectoderm cells mixed with non-competent late gastrula ectoderm or endoderm, which primarily form common aggregates, do not differentiate into neural derivatives. In these reaggregates the ectoderm cells remain separated from each other by heterologous cells (non-competent ectoderm or endodermal cells) during the period of competence. These data indicate that the quick recovery of extracellular matrix components together with the restoration of the former organization of the plasma membrane is responsible for the prevention of neuralization.

Extracellular matrix; Glycocalyx; Inhibition of neural differentiation; *Xenopus laevis*; Neural induction

The paper, which already in 1990 suggested the existence of a factor at the cell surface of the ectoderm, which prevents neural differentiation. *Wilson and Hemmati-Brivanlou identified this molecule 5 years later as BMP-4.*

In the traditional view it has been suggested that neuralizing factors play an instructive role resulting in the formation of the neural plate. In this model the ectodermal cells should contain specific receptors to interact with the neuralizing factors. However, recent data could show that neuralizing factors form complexes with BMP-4 in the extracellular space between inducing chordamesoderm and reacting neuroectoderm. This interaction prevents the binding of BMP-4 to its receptor, which results in the neuralization of the ectoderm (Sasai *et al.,* 1994,1995; Bouwmeester *et al.*, 1996; Piccolo *et al.*, 1996). Of central importance is the fact that apparently several factors must interact with each other during the determination of the anterior posterior pattern formation. Furthermore the interaction of enzymes with the chordin-BMP4-complex is considered as an important factor for anterior-posterior gradient formation (Piccolo *et al.*, 1997). Head structures can only be formed if simultaneously BMP-4, Wnt-8 and nodal interacts with *cerberus*, a gene expressed in the most anterior part of the involuting endomesoderm (Piccolo *et al.*, 1999). Similar mechanisms could be observed with *dickkopf*, a gene also expressed in the Spemann-Mangold organizer area (Glinka *et al.*, 1998).

Signalling during the primary steps of neural induction

Although the knowledge about embryonic axis formation and the participating genes and their products has exponentially increased during the last 5 years, the exact mechanism of neural induction and determination of the anterior/posterior polarity of the central nervous system was the target for controversial discussions. It has been postulated that planar signals migrating from the Spemann-Mangold organizer to the neighbouring ectoderm should be sufficient for an anterior/posterior determination of the presumptive central nervous system (Doniach *et al.*, 1992; Ruiz i Altaba, 1992). In contrast Holtfreter (1933d) could show with so-called "total exogastrulae" that vertical signals during the involution of chordamesoderm are most important for the early steps of neural determination. Using a special technique to prepare experimentally exogastrulae (so-called pseudoexogastrulae) and the new molecular methods we could support the view that planar signals are of minor importance during the early steps of neural determination in both Triturus (urodela) and *Xenopus* (anura)(Chen *et al.*, 2000).

Still open questions - intracellular signalling, competence, and homogenetic induction

On the basis of data with neuralized ectoderm after treatment at high pH Holtfreter postulated that a neuralizing factor should be present in the competent ectoderm in an inactive masked form (Holtfreter, 1934 a, 1934b). This idea was supported by more recent experiments (John *et al.*, 1984). Therefore at present it cannot be decided, if the secreted factors emanating from the dorsal mesoderm and antagonize BMP-4, are the neuralizing factors proper or if these secreted factors activate an intracellular neuralizing factor. Similar mechanisms apply to homoigenetic induction, i.e. induction of ectoderm into neural tissue by interaction with neural plate (Mangold, 1933). The factor secreted from the neural plate can be an inducing factor proper, but a releasing factor for the neuralizing factor in the ectoderm is so far not excluded (Tiedemann *et al.*, 1998).

Ectoderm of early gastrulae of Triturus alpestris differentiates into neural structures (56% of the cases) after treatment with 75 nM phorbol ester. Comparable effects can be observed with *Xenopus* laevis ectoderm, when treated with slightly higher concentrations (Davids *et al.*, 1987). Phorbol ester activates protein kinase C in many organisms. In amphibians the activity of protein kinase C will be increased, when gastrula stages of *Xenopus* laevis are treated with neuralizing factor (Davids, 1988; Otte *et al.*, 1988, 1990). L-type Ca^{++}-channels are under the control of protein kinase C, which is activated by phorbol ester. The intracellular Ca^{++}-concentration rises transiently after treatment of ectoderm with phorbol ester or the lectin concanavalin A, which also evokes neuralization (Takata *et al.*, 1981; Grunz, 1985). These data suggest that Ca^{++}-channels, which mediate Ca^{++}-transport, are activated by neural induction (Drean *et al.,* 1994; Moreau *et al.*, 1994). Although there exists a cross-talk between protein kinase C and cyclic AMP, cAMP has no neuralizing activity for its own (Grunz and Tiedemann, 1977; Otte *et al.*, 1989).

Furthermore the exact molecular mechanism of the so-called competence is still obscure. Ectoderm after the loss of competence during the course of gastrulation will no longer react on inducing factors even in high concentrations. In an early paper we could show that lithium chloride induces mesodermal structures in Triturus alpestris and Axolotl ectoderm in high percentage in the middle blastula (Grunz, 1968). This is in agreement with the high reactivity of ectoderm in the middle blastula to activin. Furthermore we could show that protein synthesis is essential for the loss of competence of ectodermal target cells during gastrulation (Grunz, 1970). Rupp and co-workers could show that apparently somatic linker histones

could play an important role in the loss of mesodermal competence (Steinbach *et al.*, 1997).

Conclusions and perspectives

Using molecular genetic techniques our knowledge about the processes during ontogenesis and evolution has dramatically improved in the last 5 years. Comparative studies with invertebrates and vertebrates have shown that many embryonic processes on the level of gene regulation and pattern formation are evolutionary conserved. Exactly 60 years after Spemann, three Developmental ("Molecular") Biologists received the Nobel prize in 1995 in Experimental Medicine and Physiology, which shows that there exist today a close correlation between Embryology, Cell Biology, Developmental Biology (Physiology), molecular and classical Genetics and Evolution. In fact in many labs of Developmental (Geneticists) Biologists the strategies and methods of all research fields mentioned above are used simultaneously. By molecular techniques could be shown that homologous genes and their products are expressed in different animal phyla. These results opened new perspectives ranging from coelenterates to vertebrates in the discussion of evolutionary conserved mechanisms of ontogeny and phylogeny. Therefore traditional views in comparative anatomy and developmental biology about convergent development of organs including definitions about homology and analogy must be partially revised (Halder *et al.*, 1995a, 1995b; De Robertis and Sasai, 1996; De Robertis, 1997; Grunz, 1999b). Although a lot of studies are performed meanwhile on other vertebrates like zebrafish and mice, the amphibian embryo is still the most favourable vertebrate model system for many topics. Also transgenic *Xenopus* can be produced (Amaya and Kroll, 1999). Furthermore *Xenopus* tropicalis because of its short generation time and diploid genome will be superior to *Xenopus* laevis in several aspects. Recently could be shown that the amphibian embryo is a powerful model system for organ engineering (Grunz, 1999a; Chan *et al.*, 1999). The amphibian ectoderm is an omnipotent germ layer and will differentiate into derivatives of all germ layers and tissues (Grunz, 1983). Therefore it could be compared with the mammalian omnipotent stem cells and will be also in the next future a valuable tool to study the basic mechanisms of tissue and organ engineering.

Summary

After the Hans Spemann and Hilde Mangold discovery of the importance of the dorsal blastopore lip for axis formation in the early embryo (Nobelprize for Spemann, 1935), the scientific community tried in a goldrush-like manner to find the inducing factors responsible for the programming of early embyronic determination and differentiation. The slow progress towards a solution of this problem caused a fading of interest on behalf of most laboratories. This article describes the activities of a few laboratories in Finland, Japan and Germany, which continued their studies despite tremendous experimental difficulties. Finally only Heinz Tiedemann's group in Berlin was the first which could isolate a mesoderm/endoderm inducing factor in highly purified form, the so-called vegetalizing factor, now known as activin. Furthermore this article describes the identification of neuralizing factors like Chordin, Cerberus and Dickkopf in the zone of the Spemann-Mangold organizer. The finding that BMP-4 acts as an antagonist to these factors located on the dorsal side led to a new understanding of the mechanisms of action of inducing (neuralizing) factors and early embryonic pattern formation. Moreover, the observations that closely related genes and their products were also found in *Drosophila*, Zebrafish, Mice and Human were the basis for new concepts of evolutionary mechanisms (dorsal/ventral and anterior/posterior polarity or conserved processes in eye-development of all 7 animal phyla).

Acknowledgements

The work of the author was supported by grants from the Deutsche Forschungsgemeinschaft and in part by the Forschungspool of the Universität Essen.

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